Luminescent Cell Health Assays for Tumor Spheroid Evaluation

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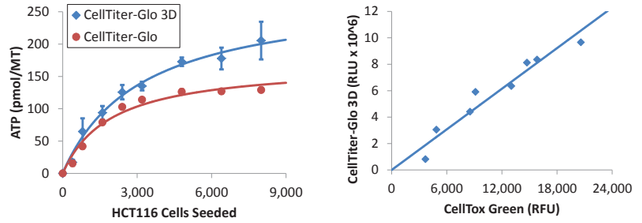
1. Introduction

Microtissues produced in 3D cell culture are much more representative of actual living tissue compared to monolayers produced in 2D cell culture. In fact, in the area of oncology research, multicellular tumor spheroids are considered an excellent platform for testing drug delivery and efficacy. As the necessity for established 3D cell culture models rises, there is also a need for convenient assays that have been specifically demonstrated to be effective for use with 3D microtissues. The more complex architecture of 3D microtissues demands increased lytic effectiveness and reagent penetration, characteristics that are often only minor considerations for reagents designed for 2D cell culture. Here we report on a variety of bioluminescent and fluorescent cell-based assays applied to hanging-drop spheroids produced from HCT116 colon cancer cells. The first assay to be described is an ATP detection reagent for measuring cell viability. This reagent has both an improved formulation and an optimized assay protocol and has clear advantages over other viability assays. Other cell health assays will also be described, including reagents that measure cell death, apoptosis, mechanistic cytotoxicity, or reporter gene expression. These additional assays do not require a change in formulation, but do require new protocols in order to optimize their effectiveness when applied to 3D microtissues. As with their application to cells in 2D culture, these “add-mix-measure” reagents are robust and amenable to both low- and high-throughput applications.

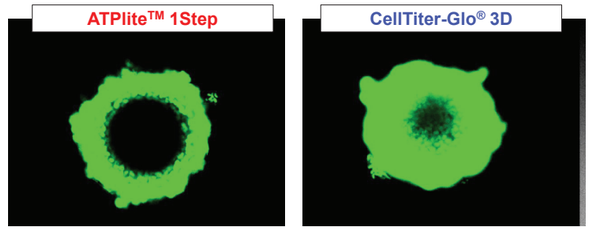
2. CellTiter-Glo® 3D – an improved viability reagent



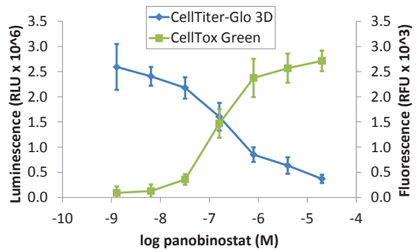
HCT116 cells (RPMI +10% FBS) were seeded in InSphero’s GravityPLUS™ 96-well hanging-drop platform and grown for 4 days. Microtissues were assayed by adding an equal volume of reagent, shaking for 5 minutes, and recording luminescence or fluorescence after 30 minutes. (Left) The ATP per microtissue is greater for the new 3D reagent compared to the classic reagent. (Right) By including 2x CellTox™ Green in CellTiter-Glo® 3D prior to sample addition, a linear correlation is observed between the ATP detected by CellTiter-Glo® 3D and the DNA detected by CellTox™ Green.

3. CellTiter-Glo® 3D – better reagent penetration

400 HCT116 cells (RPMI +10% FBS) were seeded in InSphero’s GravityPLUS™ 96-well hanging-drop platform and grown for 4 days to generate ~300 um diameter spheroids. CellTox™ Green, a membrane-impermeant dye that binds DNA, was added to each reagent prior to sample addition. After 5 minutes of shaking, confocal laser fluorescent microscopy images were obtained ~30 min after reagent addition.

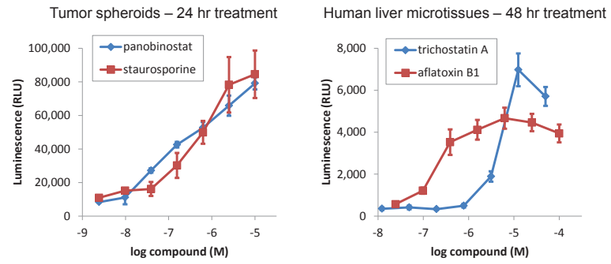


4. CellTox™ Green – cytotoxicity assay



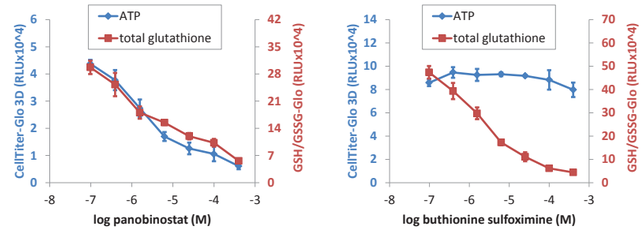
HCT116 cells (RPMI +10% FBS) were seeded in InSphero’s GravityPLUS™ 96-well hanging-drop platform and grown for 4 days to form ~350 μm spheroids. Microtissues were treated with panobinostat for 48 hours in the presence of 1x CellTox™ Green. After recording fluorescence, the microtissues were assayed by adding an equal volume of CellTiter-Glo® 3D, shaking for 5 minutes, and recording luminescence after 30 minutes.

5. Caspase-Glo® 3/7 – detection of apoptosis



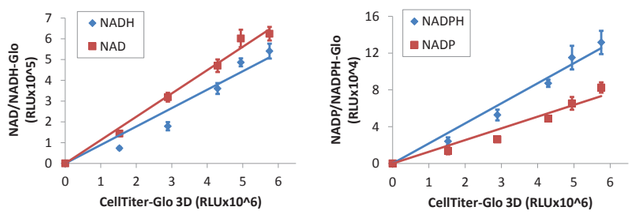
HCT116 cells (RPMI +10% FBS) were seeded in InSphero’s GravityPLUS™ 96-well hanging-drop platform and grown for 4 days to form ~340 μm spheroids. Human liver microtissues (~275 μm) were provided by InSphero. After treatment, all microtissues were assayed by adding an equal volume of Caspase-Glo® 3/7 and recording luminescence after 30 minutes of shaking.

6. GSH/GSSG-Glo™ – mechanistic toxicity



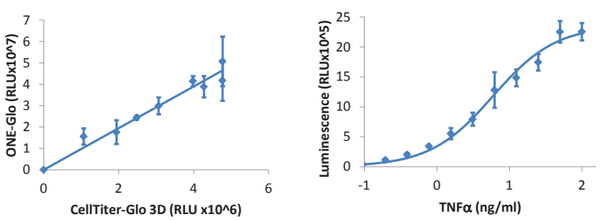
HCT116 cells (RPMI +10% FBS) were seeded in InSphero’s GravityPLUS™ 96-well hanging-drop platform and grown for 4 days to form ~350 μm spheroids. Microtissues were treated with compounds for 48 hours. For CellTiter-Glo® 3D , the microtissues were assayed by adding an equal volume of reagent, shaking for 5 minutes, and recording luminescence after 30 minutes. For GSH/GSSG-Glo™, the microtissues were assayed by removing media, adding 50 μl of total glutathione lysis reagent, and shaking for 30 minutes. Then 50μl of luciferin generation reagent was added, and after a 30 minute incubation, 100 μl of luciferin detection reagent was added. Luminescence was recorded after a final 15 minute incubation.

7. NAD/NADH-Glo™ and NADP/NADPH-Glo™ – key cellular energy metabolites



The sample generation and CellTiter-Glo® 3D assay are the same as panel 2. Media was removed from microtissues and replaced with 50 μl PBS prior to assay. For NAD/NADH-Glo™ and NADP/NADPH-Glo™ Assays, microtissues of increasing size were lysed by adding 50 μl of bicarbonate buffer, pH 10.5, + 2% DTAB and shaking for 30 minutes. After lysis, the samples were divided into 50 μl aliquots for selective dinucleotide degradation. Acid (25 μl of 0.4N HCl) was added to one aliquot, and and all were heated at 65^{0}C for 15 minutes, cooled for 10 minutes, and then neutralized with either 25 μl of 0.5M Trizma (acid-treated) or 50 μl of 0.2N HCl/0.25M Trizma (base-treated). Neutralized samples were divided and incubated with 50 μl of either the NAD/NADH-Glo™ or NADP/NADPH-Glo™ Detection Reagent. Luminescence was recorded at 60 minutes.

8. ONE-Glo™ – reporter activity



HEK293 cells (DMEM +10% FBS) were seeded in InSphero’s GravityPLUS™ 96-well hanging-drop platform and grown for 4 days. Microtissues were assayed by adding an equal volume of reagent, shaking for 10 minutes, and recording luminescence after 30 minutes. (Left) Cells expressing firefly luciferase under a constitutive reporter were seeded to generate microtissues of different size and then assayed individually with CellTiter-Glo® 3D and ONE-Glo™. (Right) After 7 hours of induction, ~340 um microtissues expressing firefly luciferase under an NFkB reporter were assayed with ONE-Glo™.

9. Conclusion

A number of luminescent assays have been successfully applied to 3D microtissues for detection of:

- ATP for cell viability (**CellTiter-Glo® 3D**)
- DNA staining of dead cells (**CellTox™ Green**)
- Caspase as a marker of apoptosis (**Caspase-Glo® 3/7**)
- Glutathione as a marker of oxidative stress (**GSH/GSSG-Glo™**)
- Nicotinamide adenine dinucleotides as markers for the metabolic state of the cell (**NAD/NADH-Glo™ and NADP/NADPH-Glo™**)
- Luciferase reporter expression (**Steady-Glo®**, **ONE-Glo™**, **Nano-Glo®**, etc.)

Are there other assays you would like to see applied to 3D microtissues?
Or are there other 3D culture systems you’d like to see these assays applied to?

For other questions or additional information, contact mike.valley@promega.com